ELSEVIER

Clinical *Sarcocystis neurona*, *Sarcocystis canis*, *Toxoplasma gondii*, and *Neospora caninum* infections in dogs

J.P. Dubey [a,*], Jennifer L. Chapman [b], Benjamin M. Rosenthal [a],
M. Mense [b], Ronald L. Schueler [c]

[a] *United States Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Animal Parasitic Diseases Laboratory, Beltsville Agricultural Research Center, Building 1001, Beltsville, MD 20705-2350, USA*
[b] *Department of Veterinary Pathology, Armed Forces Institute of Pathology,14th & Alaska Avenue, NW, Washington, DC 20306, USA*
[c] *South Carroll Veterinary Hospital, 6220 Georgetown Boulevard, Eldersburg, MD 21784, USA*

Abstract

Sarcocystis neurona, *Sarcocystis canis*, *Toxoplasma gondii*, and *Neospora caninum* are related apicomplexans that can cause systemic illness in many species of animals, including dogs. We investigated one breeder's 25 Basset Hounds for these infections. In addition, tissues from dogs and other non-canine hosts previously reported as *S. canis* infections were studied retrospectively. Schizonts resembling those of *S. neurona*, and recognized by polyclonal rabbit anti-*S. neurona* antibodies, were found in six of eight retrospective cases, as well as in two additional dogs (one Basset Hound, one Springer Spaniel) not previously reported. *S. neurona* schizonts were found in several tissues including the central nervous system, lungs, and kidneys. Fatal toxoplasmosis was diagnosed in an adult dog, and neosporosis was diagnosed in an adult and a pup related to the one diagnosed with *S. neurona*. No serological reactivity to *S. neurona* antibodies occurred when *S. canis*-like liver schizonts were retrospectively assayed from two dogs, a dolphin, a sea lion, a horse, a chinchilla, a black or either of two polar bears. Sequencing conserved (18S) and variable (ITS-1) portions of nuclear ribosomal DNA isolated from the schizont-laden liver of a polar bear distinguished it from all previously characterized species of *Sarcocystis*. We take this genetic signature as provisionally representative of *S. canis*, an assumption that should be tested with future sequencing of similar liver infections in other mammalian hosts. These findings further extend the uncharacteristically broad intermediate host range for *S. neurona*, which also causes a neurologic disease in cats, mink, raccoons, skunks, Pacific harbor seals, ponies, zebras, lynxes, and sea otters. Further work is necessary to delineate the causative agent(s) of other cases of canine sarcocystosis, and in particular to specify the attributes of *S. canis*, which corresponds morphologically to infections reported from wide range of terrestrial and marine mammals.
Published by Elsevier B.V.

Keywords: *Sarcocystis neurona*; Dogs; Schizonts; Immunoreactivity; Electron microscopy; *Sarcocystis canis*; *Toxoplasma gondii*; *Neospora caninum*

* Corresponding author. Tel.: +1 301 504 8128; fax: +1 301 504 9222.
  E-mail address: jdubey@anri.barc.usda.gov (J.P. Dubey).

1. Introduction

Sarcocystis neurona, *Sarcocystis canis*, *Toxoplasma gondii*, and *Neospora caninum* are related apicomplexans that can cause systemic illness in many species of animals, including dogs (Dubey and Beattie, 1988; Dubey and Speer, 1991; Dubey et al., 2001b; Dubey, 2003). Opossums serve as the definitive host for *S. neurona* increasingly notable for its unusually broad distribution of intermediate or aberrant hosts including horses, raccoons, armadillos, cats, sea otters and skunks. In intermediate hosts, only asexual stages are found and they are confined primarily to the central nervous system (CNS). *S. neurona* causes a fatal neurologic disease in horses (equine protozoal myeloencephalitis, EPM) and EPM-like infections have been reported in raccoons, domestic cats, a Canadian lynx, mink, skunks, a pony, a zebra, a fisher, Pacific harbor seals, and sea otters (Dubey et al., 2001b, 2002, 2003a; Forest et al., 2001; Gerhold et al., 2005). *S. neurona*-like infection was reported in a monkey (Klumpp et al., 1994) but the identification was not confirmed by *S. neurona* immunohistochemistry (Dubey and Hamir, 2000). Clinical EPM infections in animals have been reported from USA, Canada, Brazil, and Panama.

Canine sarcocystosis has previously been attributed to *S. canis*, a taxon currently defined only by morphological criteria, whose schizonts have also been diagnosed in the livers of a sea lion (Mense et al., 1992), a horse (Davis et al., 1999), two polar bears (Garner et al., 1997), a black bear (Zeman et al., 1993), a chinchilla (Rakich et al., 1992), a dolphin (Resendes et al., 2002), and a Hawaiian monk seal (Yantis et al., 2003). The schizont structure reported for *S. canis*

resembles that of *S. neurona* (Dubey and Speer, 1991), and its life cycle has not been completed. In non-canine hosts, only the schizont stage has been identified, occurring only in the liver. All but two reports of *S. canis* infections in animals were from USA (listed in the present study), one report was from a dog from Costa Rica (Berrocal and Lopez, 2003), and one was in a dolphin from Spain (Resendes et al., 2002).

Clinical, immunohistochemical, ultrastructural and genetic data have recently implicated *S. neurona* infection in a dog suffering from myositis (Vashisht et al., 2005). We were therefore curious to use immunohistochemical criteria to determine whether *S. neurona* occurred in any of two new, or eight retrospective cases of canine sarcocystosis presumed to have been caused by *S. canis* (Table 1). We characterized and compared a portion of the 18S ribosomal DNA and the entire ITS-1 of a polar bear isolate of *S. canis* in order to evaluate its phylogenetic position with respect to *S. neurona* and other congeners. Additionally, we confirmed that parasites reported by Vashisht et al. (2005) correspond, according to these genetic criteria, to *S. neurona*. Finally, we documented primary toxoplasmosis and neosporosis in a group of Basset Hounds.

2. Materials and methods

2.1. Dogs in the present study

2.1.1. Dog no. 1

A 9-year-old intact male Springer Spaniel presented with a 2-week history of left rear limb

Table 1
Sarcocystis neurona and *S. canis*-like infections in dogs

Dog no., location	Tissues	*S. neurona* staining	Reference	
1, Maryland	Brain	Positive	Present study	
2, Maryland	Brain, spinal cord	Positive	Present study	
Maryland	Liver	Negative	Dubey et al. (1991b)	
Illinois	Skin, others	Positive	Dubey et al. (1991a)	
Illinois	Brain	Positive	Dubey and Slife (1990)	
Lousiana	Lung, kidney, spleen, liver	Positive	Dubey et al. (1992)	
Kansas	Liver	Negative	Trasti et al. (1999)	Dog no. 1
Illinois	Lung	Positive		Dog no. 2
Oklahoma	Brain	Positive		Dog no. 3
Arkasas	Brain	Positive		Dog no. 4

lameness. Concurrent medical problems included a 5-year history of seizures controlled with phenobarbital treatment. The owners reported the dog as current on vaccines and receiving monthly heartworm preventative. On physical examination, the dog was non-weight bearing on the left rear limb, had atrophy of the thigh muscles, and a fever of 105.3 °F (40.2 °C). A complete blood count revealed a regenerative anemia [red blood cell count 1.29 million/mm^3 (reference range, 4.8–9.3 million/mm^3), hemoglobin 3.9 g/dl (reference range, 12.1–20.3 g/dl), hematocrit 10.5% (reference range, 36–60%), reticulocyte 9.5% (reference range, 0.2–1.1%), with anisocytosis, polychromasia, and macrocytosis]. A diagnostic workup revealed a normal coagulation profile, the dog was heartworm negative, and had negative Lyme Disease, Rocky Mountain Spotted Fever, and had no *Ehrlichia canis* antibodies. The differential diagnosis included autoimmune hemolytic anemia, infection, and neoplasia. The dog was treated with azathioprine, prednisone, and doxycycline, but remained unresponsive. An ultrasound of the liver revealed possible neoplasia. The dog continued to decline and the owners elected euthanasia in August 2004. At necropsy, firm miliary nodules were present in the lungs and no gross lesions were noted in the liver. A partial necropsy was performed and the lung, liver, spleen, and bone marrow were taken for histopathology. Specimens were submitted to the Armed Forces Institute of Pathology, Washington, DC for diagnosis.

2.1.2. Dog no. 2

An 8-month-old, male Basset Hound was necropsied 23 December 2002. The cerebrum, cerebellum, brain stem and 3 cm of cervical spinal cord were removed in toto, trimmed in the non-fixed state and placed in 10% neutral buffered formalin.

2.1.3. Dog no. 3

This 2-year-old Basset Hound was half brother to dog no. 2. This dog had seizures a few days before death. The dog was necropsied at the Veterinary Referral Associates, Gaithersburg, MD.

2.1.4. Dog nos. 4–26

One of us (JPD) was contacted by the original breeder of the Basset Hounds because dog nos. 2, and 3 had died of toxoplasmosis-like illness, not recognized earlier in the establishment. The breeder arranged to

have serum samples from 20 Basset Hounds that were related to dog nos. 2 and 3.

2.1.5. Dogs published previously as S. canis

Paraffin blocks from previously published cases from dogs were studied immunohistochemically (Table 1).

2.1.6. Dog reported by Vashisht et al. (2005)

Histologic sections of tissues from the dog and DNA extracted from the frozen muscle of the dog were further evaluated.

2.2. Non-canine hosts previously published as S. canis

Paraffin blocks from livers of the sea lion (Mense et al., 1992), the horse (Davis et al., 1999), two polar bears (Garner et al., 1997), the black bear (Zeman et al., 1993), the chinchilla (Rakich et al., 1992), the dolphin (Resendes et al., 2002), and the Hawaian monk seal (Yantis et al., 2003) were restudied immunohistochemically.

2.3. Serological examinations

Serum samples from dogs were tested for antibodies to *T. gondii* using the modified agglutination test (MAT; Dubey and Desmonts, 1987) and for *N. caninum* antibodies by the *Neospora* agglutination test (NAT; Romand et al., 1998). Sera were diluted two-fold starting at 1:10 serum dilution for *T. gondii* and 1:20 serum dilution for *N. caninum*.

2.4. Histopathologic examination

Tissues were fixed in 10% neutral buffered formalin, and processed for routine histology. Paraffin-embedded sections were cut at 5 μm, stained with hematoxylin and eosin (H and E) and examined microscopically.

2.5. Immunohistochemical examination

Deparaffinized sections were reacted with antibodies to *T. gondii*, *N. caninum*, and *S. neurona* as described by Dubey et al. (2001a). The specificity and preparation of antibodies to *S. neurona* (Dubey et al.,

1999), *N. caninum* and *T. gondii* (Lindsay and Dubey, 1989) were as described.

2.6. Transmission electron microscopy

A portion of the lung of dog no. 1 was deparaffinized and processed for transmission electron microscopy.

2.7. Molecular systematics

DNA from the liver of a polar bear that had died of acute hepatitis associated with *S. canis* (Garner et al., 1997) was extracted by proteinase K digestion, and purification using Qiagen DNAeasy columns according to the manufacturer's protocol; the liver had been stored for 10 years at −20 °C or lower. Aliquots along with extraction negatives, were subsequently used as templates in PCR assays to characterize a 994 bp portion of 18S ribosomal DNA using previously described methods (Dubey et al., 2004). These products were directly sequenced on an ABI 3100 fluorescent sequencer using BigDye 3.1 terminator chemistries. A 1116 bp portion of rDNA, incorporating the entire ITS-1 locus as well as portions of the flanking 18S and 5.8S genes, was amplified with primers 18S14-F (AGTGTTCCGGTGAATTATTC) and 5.8S-R (TTCGCTGCGTT). Three clones of each of two independent PCR products, cloned using the TOPO TA cloning kit (Invitrogen), were sequenced. Sequence contigs were assembled and edited using ContigExpress (VectorNTI, Informax Inc.).

To place the DNA from dog V within a comparative genetic context, we PCR amplified and directly sequenced a 459 bp fragment of ribosomal DNA to all available homologues. The fragment included terminal portions of 18S and 5.8S genes, and comprised the entire first internal transcribed spacer (ITS-1). Homologues were identified by BLAST, aligned using CLUSTALW, and phylogenies reconstructed from 500 bootstrap replicates under the minimum evolution criterion employing Kimura 2-Parameter distances and assuming gamma distributed variation with a shape parameter of 0.5 using MEGA 3.1 (Kumar et al., 2004).

BLAST was used to identify publicly available homologues, which (for 18S) were aligned using CLUSTALW and compared by means of 500 bootstrap replicate distance trees reconstructed from Kimura 2-parameter distances under the criterion of Minimum Evolution, as implemented by MEGA version 2.0 (Kumar et al., 2004). The phylogenetic position of *S. canis* was placed in a broad comparative framework by inclusion of other available sequences identified by a BLAST search of GenBank; by limiting a subsequent analysis to the taxa to which *S. canis* appears most closely related, we more precisely defined its phylogenetic position.

3. Results

3.1. Lesions

3.1.1. Dog no. 1
Examination of the lung and liver revealed diffuse, necrotizing interstitial pneumonia and multifocal necrotizing periportal hepatitis (Fig. 1). The inflammatory infiltrate in both the lung and liver was composed predominantly of neutrophils, macrophages, and multinucleated giant cells. Within the lung and liver, there were variable numbers of intrahistiocytic protozoa (Fig. 1B and D). In addition, lymphoid depletion in the spleen and hypocellular bone marrow were observed microscopically.

3.1.2. Dog no. 2
Gross pathologic changes were noted in the brain, lung and urinary bladder. On opening the calvarium, leakage of clear, viscous cerebrospinal fluid (CSF) was noted suggesting increased volume, fluid under pressure, or both. The cerebral gyri appeared swollen with slight flattening and sulci were shallow. The dog had a marked atrophy of the masseter and temporal muscles, and bilateral ectropion with mucoid discharge. Oral mucous membranes were pale. The trachea, primary and secondary airways contained abundant pink froth suggesting pulmonary edema. Blood stained froth could be expressed from the cut surfaces of the lung lobes. The urinary bladder was greatly distended with urine and the body was diffusely reddened. Heart and its chambers, valves and outflow tracts, mediastinum, thymus, pericardium, diaphragm, stomach, small and large bowel, mesentric lymph nodes, pancreas, liver, gall bladder (including common bile duct), kidneys and adrenal glands were normal.
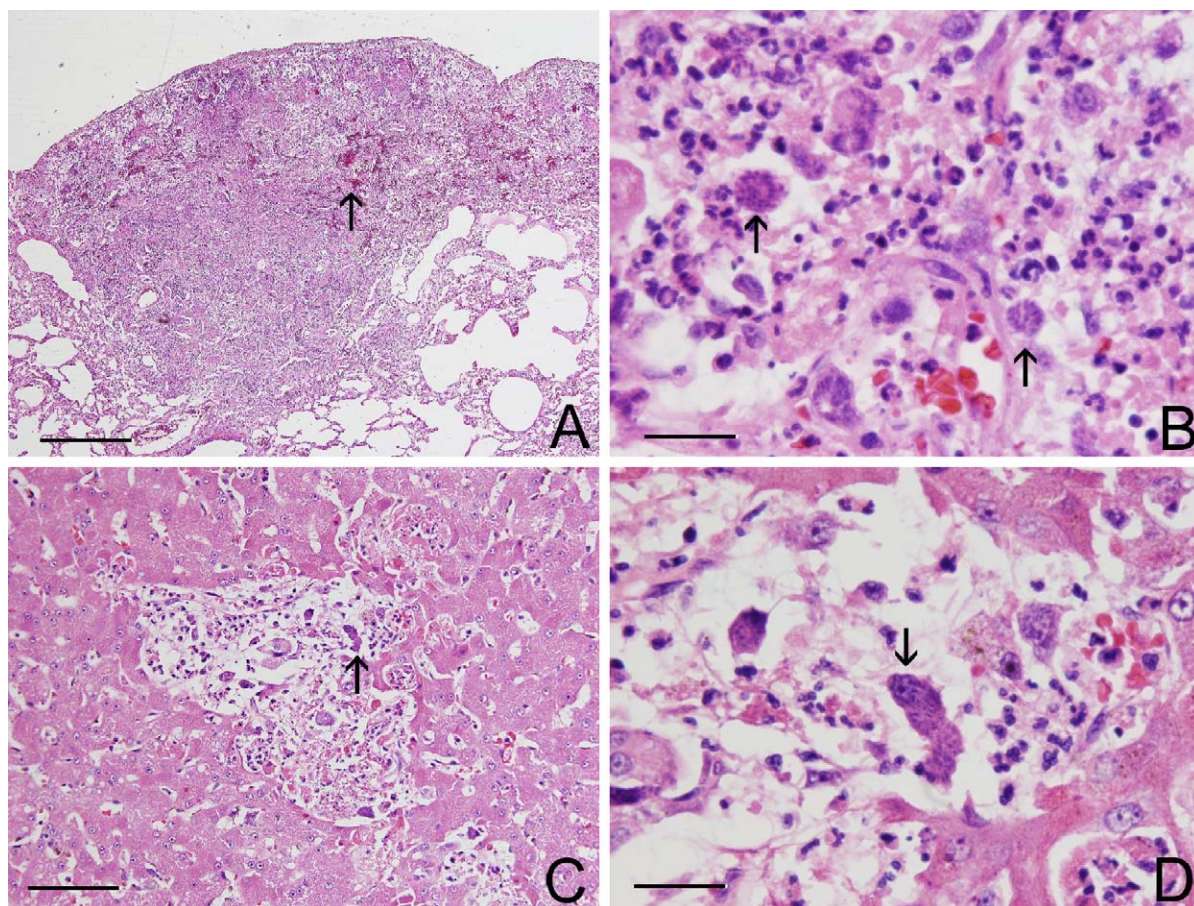
Fig. 1. Histological sections of lung (A and B) and liver (C and D) from dog no. 1. H and E stain. (A) Focal subpleural consolidation (arrow). Bar = 300 μm. (B) Schizonts (arrows) in alveoli among inflammatory cells. Bar = 15 μm. (C) Focal necrosis and a schizont (arrow). Bar = 60 μm. (D) A schizont (arrow) among degenerating inflammatory host cells. Bar = 15 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Microscopic lesions were confined to all regions of the brain and spinal cord and were characterized by marked perivascular lymphocytic/plasmacytic cuffing which included the meninges, central chromatolysis of many neurons throughout the cerebrum and neovascularization (Fig. 2A and B). Focal malacia was found in the foci of more intense inflammation. A severe loss of Purkinje cells was evident in the cerebellum along with marked inflammation of the folia. Demyelination occurred focally in fiber tracts, especially in cervical spinal cord and included swollen axon sheaths, swollen eosinophilic axons and macrophage activity. Protozoal schizonts and merozoites were present in lesions.

3.1.3. Dog no. 3

Gross lesions were seen in the lung and brain. All lung lobes had dark red firm nodules. Specimens of brain, liver, lung, spleen, sternal lymph node, myocardium, and skeletal muscle were submitted for histopathologic evaluation to the Laboratory of Veterinary Pathology, School of Veterinary Medicine, University of Pennsylvania, PA. On cut sections, there were three grossly visible lesions in the brain; two 1.5 cm tan-red nodules were in the lateral mid-cerebral cortex and the third 1 cm lesion was in the dorsal occipital area.

Microscopically, main lesions were in the brain and the lung. The encephalitis was characterized by severe multifocal necrosuppurative and lymphoplasmacytic
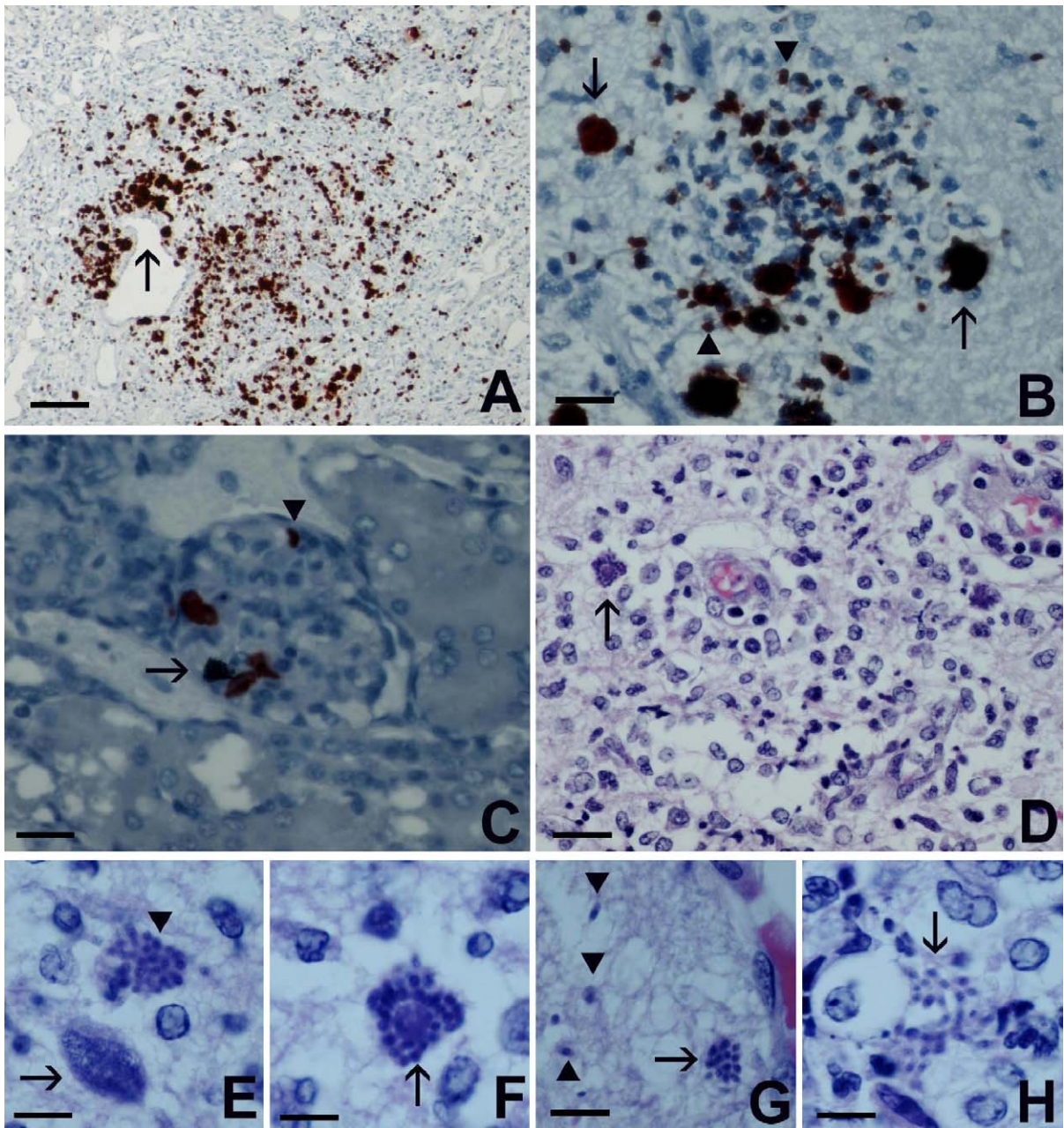
Fig. 2. Lesions and *S. neurona*-like organisms in histological sections of dogs. (A–C) Immunohistochemical staining with *S. neurona* antibodies; (D–H) H and E stain. Bar in A = 100 μm, bar in B–D = 25 μm, bar in E–H, 10 μm. (A) Lung of dog no. 1 with numerous (arrow) schizonts and merozoites (all red structures). (B) Cerebrum of dog no. 2 with numerous merozoites (arrowheads) and schizonts (arrows). (C) Kidney of the 2-day-old dog reported by Dubey et al. (1992) with schizonts (arrow) and merozoites (arrowhead) in a glomerulus. (D) Cerebrum of dog no. 2 with severe perivasulitis. Note a schizont (arrow) at the periphery of the lesion. (E) Cerebrum of dog no. 2 with an immature (arrow) and mature (arrowhead) schizont. (F) Cerebrum of dog no. 2 with a schizont with a residual body (arrow). (G) A schizont (arrow) and free merozoites (arrowheads). (H) Cerebrum of dog no. 2 with a ruptured schizont (arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

meningoencephalitis. Numerous *T. gondii*-like tachy-zoites and tissue cysts were present in and around lesions. The pulmonic lesions were characterized by multifocal chronic necrosuppurative granulomatous pneumonia and numerous tachyzoites were in the lesions

3.1.4. Dog no. 4

One dog (no. 4) was reported to have produced one protozoal-infected pup that died of a neosporosis-like disease at the age of 7 months (diagnosis was made histopathologically at the Diagnostic Veterinary Systems Laboratory, Toronto, Canada in 1998, personal communication with the breeder). The dog had interstitial myocarditis and hepatitis and *N. caninum*-like tachyzoites were found in cardiac myosites; no other information is available and tissues are not available for a retrospective study because the diagnostic laboratory went out of business. Dog no. 4
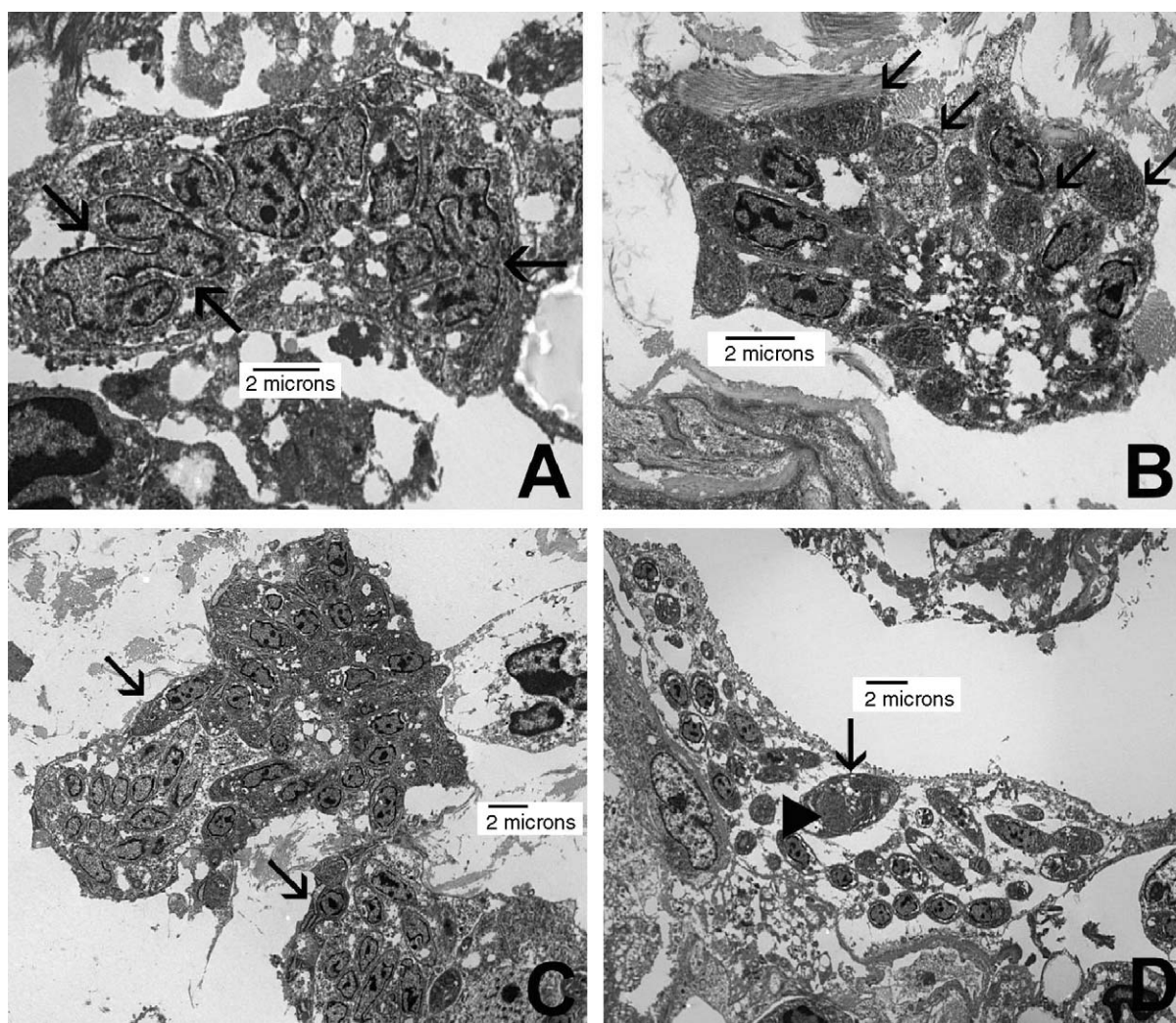

Fig. 3. Transmission electron micrographs of *S. neurona* in lung of dog no. 1. (A) Immature schizont with nucleus dividing into interconnected nuclear lobes (arrows). (B) A small schizont with merozoites developing at the periphery. Note numerous micronemes (arrows) but no rhoptries in merozoites. (C) Schizonts with developing merozoites (arrows). (D) A schizont with several merozoites one of which (arrow) is already developing into a new schizont without leaving the parent cell. Note dividing nucleus (arrowhead) of the immature schizont.

was bred only once. The breeder lives on a farm and dogs run on a large field with woods.

3.1.5. Dog no. 5

This 5-year-old female Basset Hound, from the same owner as dog no. 4, became suddenly ill and developed hind limb paralysis and weakness of the back, had a behaviour change (became viscious) and had to be confined to a cage. She died 2 months later. *N. caninum* IFAT titer performed at the Cornell University College of Veterinary Medicine, Ithaca, NY was 1:800 at the beginning of the illness and at the time of death. Histological examination revealed myeleomalacia and encephalomyelitis. Numerous protozoal tachyzoites and few tissue cysts in brain and spinal cord reacted with *N. caninum* monoclonal antibody described by Cole et al. (1994). There was no reactivity to *T. gondii* polyclonal antibodies; the immunohistochemistry was performed at the Prairie Diagnostic Services, Saskatoon, SK, Canada.

Reterospective staining of the sections of the brain and spinal cord with polyclonal antibodies to *T. gondii*, *N. caninum*, and *S. neurona* in the present study confirmed the diagnosis of neosporosis; there was no

Fig. 4. A *S. neurona* schizont with numerous merozoites (arrows) arranged haphazardly, several of them cut longitudinally (double arrows).

reactivity to *S. neurona* antibodies and only a mild reactivity to *T. gondii* antibodies.

3.2. Protozoa

Protozoal schizonts and merozoites were found in the lung and liver of dog no. 1 and in the brain and spinal cord of dog no. 2 (Fig. 1). Immature and mature schizonts, and free merozoites were identified. The residual body was eosinophilic in sections stained with H and E. Intracellular and free merozoites were scattered in lesions (Fig. 2G). The merozoites were approximately 4–5 μm long and had a central vesicular nucleus (Fig. 2G).

Ultrastructural examination revealed that the protozoa divided by endopolygeny. The schizont nucleus divided in to many interconnected nuclear lobes (Fig. 2A). The merozoites were formed internally as well at the periphery of the schizont, sometimes with a residual body (Figs. 3B–D and 4). Merozoites had numerous micronemes but no rhoptries (Fig. 5).

Tachyzoites and tissue cysts of *T. gondii* were found in the brain of dog no. 3.

3.3. Serological examination

Antibodies to *T. gondii* were not found in 1:10 serum dilution of any of the 20 Basset Hounds tested. Antibodies to *N. caninum* were found in five dogs, in titers of 1:20 in one, 1:40 in 1, 1:80 in two, and 1:160 in two dogs; all of these dogs were clinically normal.

3.4. Immunohistochemical staining

Schizonts and merozoites in dog nos. 1 and 2 stained intensely with polyclonal antibodies to *S. neurona* (Fig. 2) and did not react with antibodies to *T. gondii* and *N. caninum*. Retrospectively, protozoa in six dogs previously considered as *S. canis* stained positively with *S. neurona* antibody (Table 1). In particular, schizonts and merozoites in the lung and kidney of the 2-day-old dog from Louisiana (Dubey et al., 1992) stained strongly with *S. neurona* (Fig. 2C).

The protozoa in the liver of the horse, bear, chinchilla, sea lion, seal, dolphin, and two dogs (Table 1), previously diagnosed as *S. canis* did not react with antibodies to *S. neurona*. Protozoa in the brain of dog no. 3 reacted only to *T. gondii* antibodies.
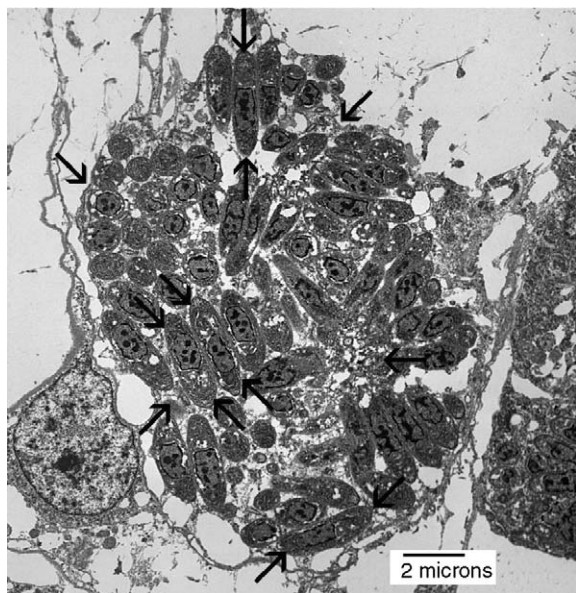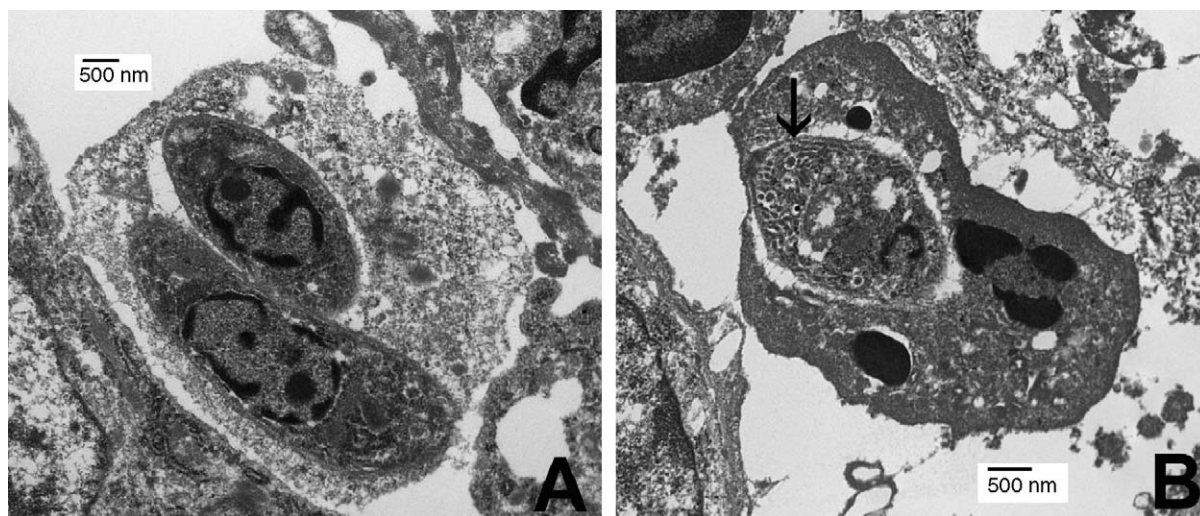
Fig. 5. Intracellular merozoites of *S. neurona.* Note presence of numerous micronemes (arrow) and absence of rhoptries.

3.5. Dog V of Vashisht et al. (2005)

Schizonts and individual merozoites were found in histological sections of lung and skeletal muscles of the dog V. The pulmonic lesions were characterized by moderate, subacute to chronic, multifocal, pyogranulomatous, fibrosing alveolitis. The schizonts appeared to be in parenchyma and not in blood vessels. In muscle sections schizonts and merozoites appeared exclusively in connective tissue between myocytes. These schizonts and merozoites stained intensely with the polyclonal rabbit *S. neurona* antibodies against culture derived merozoites as reported in Section 2.5; sarcocysts in muscles stained only mildly, or not at all, with this antibody.

3.6. Molecular systematics

Molecular systematics confirmed that the acute hepatitis occurring in the polar bear, previously diagnosed with *S. canis* (Garner et al., 1997) contained coccidian parasites closely related to, but distinct from, other species of *Sarcocystis* (and to forms of *Sarcocystis,* associated with raptorial birds, previously assigned to the genus *Frenkelia*). In particular, over the 994 bases sequenced from the small subunit ribosomal DNA, the polar bear isolates provisionally taken here to represent *S. canis* differed from *S. neurona* at six

positions (GenBank accession no. DQ146148). More generally, analysis of 18S rDNA variation supports the membership of *S. canis* within a group that also includes *S. muris*, *S. rodentifelis*, *S. neurona*, *S. dispersa*, *S. mucosa*, and species of *Frenkelia* (Figs. 6 and 7).

The ITS-1 (GenBank number DQ176645) sequence provided further means to individuate the polar bear isolate of *S. canis.* Among the several clones sequenced from two independent PCR products, four noted ''non-singleton'' conflicts were observed, which may reflect in vitro sequencing errors but which may instead represent endogenous variation among paralogous rDNA copies. Similarly, a monoadenosine repeat, beginning at base 997, varied in length from 12 to 14 residues among sequenced clones. The remaining 1112 bases were uniform among the clones, of which only the flanking portions of comparatively well-conserved 18S and 5.8 rDNA resembled (nearly perfectly) other coccidian homologues—most especially to *S. mucosa* and *S. muris*. The internal transcribed spacer itself, a non-functional intergenic locus, bore no resemblance to any other available homologue, suggesting an appreciable evolutionary divergence between it and other characterized congeners.

The 459 bp of rDNA sequenced from dog V resembled a broad array of coccidian rDNA at the
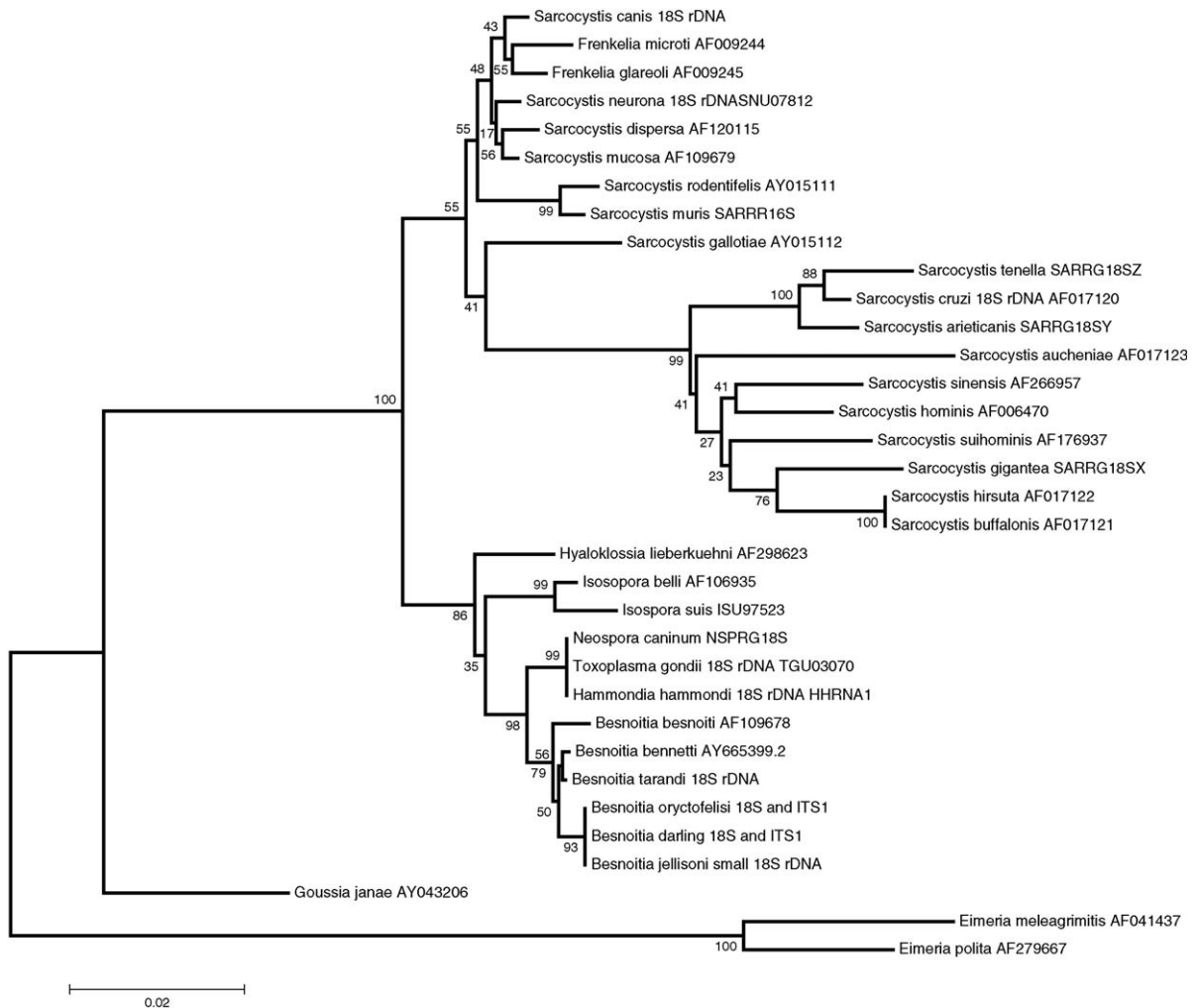
Fig. 6. Phylogeny reconstructed from 500 bootstrap replicates of partial rDNA sequences under the Minimum Evolution criterion using Kimura 2-parameter distances assuming gamma distributed rate variation with shape parameter = 0.5. The *S. canis* appears as a related, but distinct member of a clade that also comprises *S. dispersa*, *S. mucosa*, *S. neurona*, and species associated with raptorial bird definitive hosts and assigned to the genus *Frenkelia*.

strongly conserved terminus of 18S rDNA, but could be aligned at the more variable ITS-1 portion only to isolates attributed to *S. neurona*, *S. falcatula*, *S. dasypi*, and certain undefined isolates also derived from opossum hosts. Among these, phylogenetically informative variation was concentrated at a handful of nucleotide positions. Significantly, the parasite DNA derived from the canine infection described first by Vashisht et al. (2005), "Dog V", exclusively and entirely corresponded to several other exemplars of *S. neurona* (Fig. 8).

4. Discussion

We used reactivity to anti-*S. neurona* polyclonal antibodies as a criterion to diagnose *S. neurona* infections in six of eight retrospective canine cases of sarcocystosis, and in two new additional cases. Thus, some (but not all) canine cases previously attributed to *S. canis* should instead be attributed to *S. neurona*. None of several cases of sarcocystosis in a variety of other mammalian hosts, corresponding to *S. canis* morphology, similarly reacted to the anti-*S. neurona* antibodies.
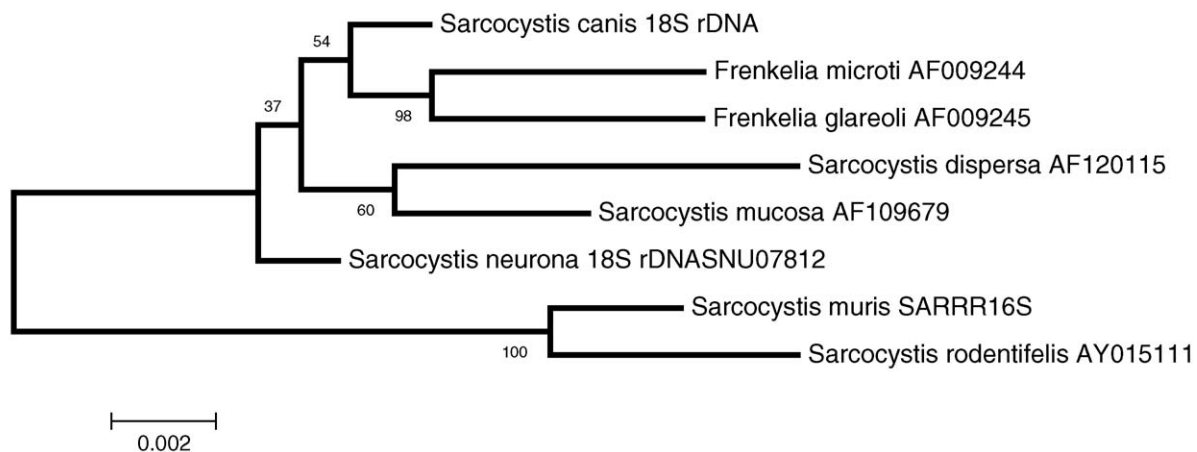
Fig. 7. Phylogeny constructed with similar methods as Fig. 6, but restricted to just those taxa to which *S. canis* apparently shares closest evolutionary affinity.
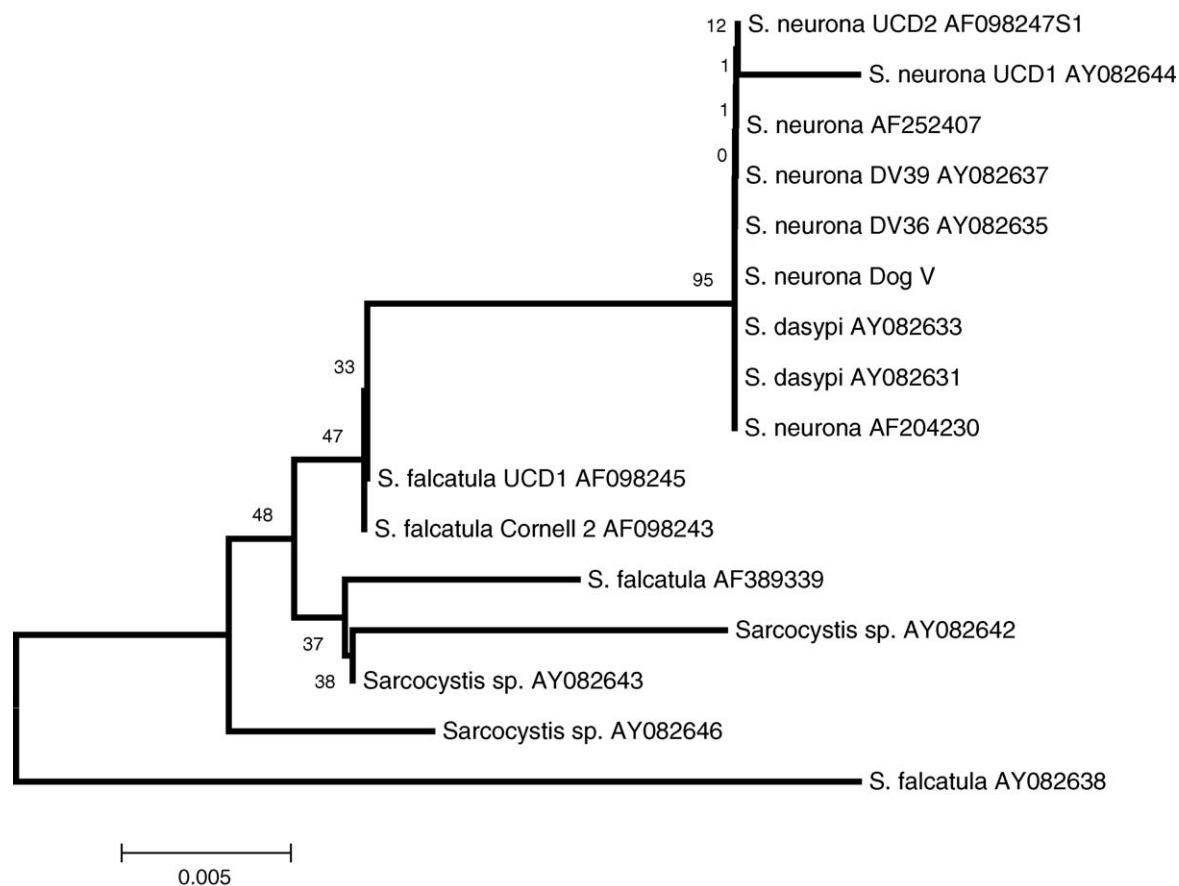
Fig. 8. Relationship of dog V to other isolates of *S. neurona* reconstructed from ITS-1 rDNA. Midpoint rooted minimum evolution tree reconstructed from Kimura 2-parameter distances and γ distributed variance across sites with shape parameter = 0.5.

At present, we lack a means to unequivocally diagnose *S. canis*, or other parasites which share the morphological attributes that also define *S. neurona*. Prior to the discovery of the *S. neurona* life cycle, *S. neurona* and *S. canis* were primarily distinguished based on tissue localization: schizonts of *S. canis* were thought to be confined to the liver and those of *S. neurona* to the CNS (Dubey and Speer, 1991). However, *S. neurona* schizonts are now known to occur in extraneural tissues (Hamir and Dubey, 2001; Dubey et al., 2001b).

Because neither the life cycle nor cell culture has been achieved for *S. canis*, no reagents are available to unequivocally diagnose *S. canis*. Our present observations, however, demonstrate that organisms previously attributed to *S. canis* in two previous canine cases, and in a variety of non-canine hosts, do not react with *S. neurona* antibodies. Additionally we provide preliminary data on molecular differences between *S. canis*-like parasite from the polar bear and *S. neurona* with a hope that this information will assist in characterizing the *S. canis* infections in dogs. It seems fair to conclude that indistinguishable schizonts of at least two, antigenically distinct organisms occur in dogs. Specifying unique genetic and serological attributes of *S. canis* would aid future clinical and epidemiological studies, and would help clarify whether infections attributed to *S. canis* in a wide range of mammalian hosts indeed correspond to a single etiological agent. The paucity of phylogenetically informative variation in the highly conserved 18S rDNA limits the statistical confidence with which we can estimate the interrelationships among these several species (low bootstrap support for many nodes within this clade), and the excess of ITS-1 variation preclude meaningful (homologous) alignment. However, the available data clearly provide the necessary means to discriminate *S. canis* from *S. neurona* in future cases of canine sarcocystosis. The distinct ITS-1 signature provides a newfound means to test the hypothesis that the liver infections of a variety of mammals are caused by a distinct parasitic taxon, *S. canis*.

Muscle tissues were not available from any of the dogs in the present study to determine if sarcocysts were present. Elsewhere, sarcocysts dissimilar to those of *S. neurona* have been identified in tissues of naturally infected dogs (Sahasrabudhe and Shah, 1966; Hill et al., 1988; Blagburn et al., 1989; Bwangamoi et al., 1993; Chapman et al., 2005). In those reports, schizonts were not found.

Vashisht et al. (2005) reported an interesting case of myositis and muscular atrophy in an immunosuppressed 2-year-old dog from Illinois, USA. Immature sarcocysts of a *S. neurona*-like parasite were found in histological sections. These sarcocysts were visible in H and E stained sections of muscles and the sarcocyst wall had villar protrusions that were reported to be ultrastructurally similar to *S. neurona*. However, the villi in the dog were much smaller ($0.75\ \mu m \times 0.25\ \mu m$, Vashisht et al., 2005) than those of *S. neurona* ($2.8\ \mu m \times 0.4\ \mu m$) mm) (Dubey et al., 2001b). Although the authors concluded that each villus contained a single microtubule that did not extend into the granular layer, resolving their true number would require better preservation. The sarcocyst did not stain with a *S. neurona*-specific monoclonal antibody, although unknown stages of the parasite in the muscle interstitium did; these immunoreactive stages were not identified further because they were not found in H- and E-stained sections. Whether the sarcocysts and the stages in the muscle interstitium were the same organism could not be determined with certainty. Although the authors speculated that the sarcocyst breakdown might have given rise to immature organisms, this phenomenon is not known to occur in any species of the genus *Sarcocystis* (Dubey et al., 1989a,b). Parasite DNA isolated from infected muscles of this dog specifically resembled that of *S. neurona*. The finding of protozoa (considered to be schizonts or merozoites) in this dog with concurrent presence of sarcocysts has not been reported earlier. We retrospectively identified schizonts and merozoites in H and E sections of lung and muscle of this "Dog V". Whether schizonts and sarcocysts belonged to the same or different parasites could not be resolved with available data. It should be noted that *S. neurona* schizonts were reported only once in extraneural tissues of all animals that died due to naturally occurring EPM-like syndrome in animals (Dubey et al., 2001b). The exception was infected heart tissue from a raccoon (Hamir and Dubey, 2001).

Gerhold et al. (2005) reported *S. neurona* associated encephalitis in a fisher confirmed by immunohistochemistry, PCR and restriction fragment length polymorphism testing, and genetic sequencing. Sarcocysts found in the muscles of this fisher were

morphologically similar to *S. neurona* sarcocysts but were negative for *S. neurona* by PCR.

The diagnosis of three diseases attributable to intracellular apicomplexan parasites among a single cohort of Basset Hounds aroused suspicion of an underlying genetic deficiency, or a common source of exposure. Clinical canine toxoplasmosis rarely results from primary infection; instead, most dogs that die of toxoplasmosis have distemper virus infection or other immunosuppressive condition (Dubey and Beattie, 1988; Dubey et al., 1989a,b, 2003b). Therefore, the death from acute toxoplasmosis was of special interest. Postnatal infection in that dog was likely, because other dogs in the establishment were not seropositive for *T. gondii*. Four of the 20 dogs tested had *N. caninum* antibodies, but these dogs had no apparent familial infection. Neosporosis was diagnosed in a 7-month-old dog; the dam had an NAT titer of 1:160. Although, *N. caninum* can be vertically transmitted in dogs, this does not appear to be epidemiologically important mode of infection (Barber and Trees, 1998; Dubey et al., 2005). The bitch that gave birth to the infected pup was spayed to prevent vertical transmission. The occurrence of three related protozoan infections in these Basset Hounds may have been coincidental. The dogs were raised on a farm and could have been exposed to *S. neurona* by ingesting grass and soil contaminated with *S. neurona* sporocysts excreted by opossums. Although the dogs were not fed raw meat, the dogs might have become infected with *N. caninum* from the environment contaminated with oocysts excreted in feces of coyotes or dogs or by ingesting tissues of other dead animals, especially deer.

5. Conclusion

The definitive diagnosis of *S. neurona* infection in dogs will require cultivation of the parasite; until now *S. neurona* has been isolated only from horses, sea otters, Pacific harbor seals, and opossums. It is clear from the current work that at least two, morphologically distinct organisms occur in dogs, one of which corresponds to *S. neurona* using serological and genetic criteria. The intermediate host range of *S. neurona*, already exceptionally broad, now clearly includes dogs. And the identity of organisms previously presumed to represent *S. canis* requires additional clarification.

References

Barber, J.S., Trees, A.J., 1998. Naturally occurring vertical transmission of *Neospora caninum* in dogs. Int. J. Parasitol. 28, 57–64.

Berrocal, A., Lopez, A., 2003. Pulmonary sarcocystosis in a puppy with canine distemper in Costa Rica. J. Vet. Diagn. Invest. 15, 292–294.

Blagburn, B.L., Braund, K.G., Amling, K.A., Toivio-Kinnucan, M., 1989. Muscular *Sarcocystis* in a dog. Proc. Helminthol. Soc. Wash. 56, 207–210.

Bwangamoi, O., Ngatia, T.A., Richardson, J.D., 1993. *Sarcocystis*-like organisms in musculature of a domestic dog (*Canis familiaris*) and wild dogs (*Lycaon pictus*) in Kenya. Vet. Parasitol. 49, 201–205.

Chapman, J., Mense, M., Dubey, J.P., 2005. Muscular sarcocystosis in a dog. J. Parasitol. 91, 187–190.

Cole, R.A., Lindsay, D.S., Dubey, J.P., Toivio-Kinnucan, M.A., Blagburn, B.L., 1994. Characterization of a murine monoclonal antibody generated against *Neospora caninum* by western blot analysis and immunoelectron microscopy. Am. J. Vet. Res. 55, 1717–1722.

Davis, C.R., Barr, B.C., Pascoe, J.R., Olander, H.J., Dubey, J.P., 1999. Hepatic sarcocystosis in a horse. J. Parasitol. 85, 965–968.

Dubey, J.P., 2003. Review of *Neospora caninum* and neosporosis in animals. Kor. J. Parasitol. 41, 1–16.

Dubey, J.P., Desmonts, G., 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. Equine Vet. J. 19, 337–339.

Dubey, J.P., Beattie, C.P., 1988. Toxoplasmosis of Animals and Man. CRC Press, Boca Raton, Florida, pp.1–220.

Dubey, J.P., Carpenter, J.L., Topper, M.J., Uggla, A., 1989a. Fatal toxoplasmosis in dogs. J. Am. Anim. Hosp. Assoc. 25, 659–664.

Dubey, J.P., Speer, C.A., Fayer, R., 1989b. Sarcocystosis of Animals and Man. CRC Press, Boca Raton, Florida, pp.1–215.

Dubey, J.P., Slife, L.N., 1990. Fatal encephalitis in a dog associated with an unidentified coccidian parasite. J. Vet. Diagn. Invest. 2, 233–236.

Dubey, J.P., Speer, C.A., 1991. *Sarcocystis canis* n. sp. (Apicomplexa: Sarcocystidae), the etiologic agent of generalized coccidiosis in dogs. J. Parasitol. 77, 522–527.

Dubey, J.P., Slife, L.N., Speer, C.A., Lipscomb, T.P., Topper, M.J., 1991a. Fatal cutaneous and visceral infection in a Rottweiler dog associated with a *Sarcocystis*-like protozoon. J. Vet. Diagn. Invest. 3, 72–75.

Dubey, J.P., Cosenza, S.F., Lipscomb, T.P., Topper, M.J., Speer, C.A., Hoban, L.D., Davis, S.W., Kincaid, A.L., Seely, J.C., Marrs, G.E., 1991b. Acute sarcocystosis-like disease in a dog. J. Am. Vet. Med. Assoc. 198, 439–443.

Dubey, J.P., Duncan, D.E., Speer, C.A., Brown, C., 1992. Congenital sarcocystosis in a two-day-old dog. J. Vet. Diagn. Invest. 4, 89–93.

Dubey, J.P., Mattson, D.E., Speer, C.A., Baker, R.J., Mulrooney, D.M., Tornquist, S.J., Hamir, A.N., Gerros, T.C., 1999. Characterization of *Sarcocystis neurona* isolate (SN6) from a naturally infected horse from Oregon. J. Eukaryot. Microbiol. 46, 500–506.

Dubey, J.P., Hamir, A.N., 2000. Immunohistochemical confirmation of *Sarcocystis neurona* infections in raccoons, mink, cat, skunk and pony. J. Parasitol. 86, 1150–1152.

Dubey, J.P., Garner, M.M., Willette, M.M., Batey, K.L., Gardiner, C.H., 2001a. Disseminated toxoplasmosis in magpie geese (*Anseranas semipalmata*) with large numbers of tissue cysts in livers. J. Parasitol. 87, 219–223.

Dubey, J.P., Lindsay, D.S., Saville, W.J.A., Reed, S.M., Granstrom, D.E., Speer, C.A., 2001b. A review of *Sarcocystis neurona* and equine protozoal myeloencephalitis (EPM). Vet. Parasitol. 95, 89–131.

Dubey, J.P., Hamir, A.N., Topper, M.J., 2002. *Sarcocystis mephitisi* n. sp. (Protozoa: Sarcocystidae) *Sarcocystis neurona*-like and toxoplasma-like infections in striped skunk (*Mephitis mephitis*) J. Parasitol. 88, 113–118.

Dubey, J.P., Benson, J., Larson, M.A., 2003a. Clinical *Sarcocystis neurona* encephalomyelitis in a domestic cat following routine surgery. Vet. Parasitol. 112, 261–267.

Dubey, J.P., Ross, A.D., Fritz, D., 2003b. Clinical *Toxoplasma gondii*, *Hammondia heydorni*, and *Sarcocystis* sp. infections in dogs. Parasitologia 45, 141–146.

Dubey, J.P., Sreekumar, C., Rosenthal, B.M., Vianna, M.C.B., Nylund, M., Nikander, S., Oksanen, A., 2004. Redescription of *Besnoitia tarandi* (Protozoa: Apicomplexa) from the reindeer (*Rangifer tarandus*). Int. J. Parasitol. 34, 1273–1287.

Dubey, J.P., Knickman, E., Greene, C.E., 2005. Neonatal *Neospora caninum* infections in dogs. Acta Parasitol. 50, 176–179.

Forest, T.W., Abou-Madi, N., Summers, B.A., Tornquist, S.J., Cooper, B.J., 2001. *Sarcocystis neurona*-like encephalitis in a Canada lynx (*Felis lynx canadensis*). J. Zoo Wildlife Med. 31, 383–387.

Garner, M.M., Barr, B.C., Packham, A.E., Marsh, A.E., Burek-Huntington, K.A., Wilson, R.K., Dubey, J.P., 1997. Fatal hepatic sarcocystosis in two polar bears (*Ursus maritimus*). J. Parasitol. 83, 523–526.

Gerhold, R.W., Howerth, E.W., Lindsay, D.S., 2005. *Sarcocystis neurona*-associated meningoencephaltis and description of intramuscular sarcocysts in a fisher (*Martes pennanti*). J. Wildlife Dis. 41, 224–230.

Hamir, A.N., Dubey, J.P., 2001. Myocarditis and encephalitis associated with *Sarcocystis neurona* infection in raccoons (*Procyon lotor*). Vet. Parasitol. 95, 335–340.

Hill, J.E., Chapman, W.L., Prestwood, A.K., 1988. Inramuscular *Sarcocystis* sp. in two cats and a dog. J. Parasitol. 74, 724–727.

Klumpp, S.A., Anderson, D.C., McClure, H.M., Dubey, J.P., 1994. Encephalomyelitis due to a *Sarcocystis neurona*-like protozoan in a rhesus monkey (*Macaca mulatta*) infected with simian immunodeficiency virus. Am. J. Trop. Med. Hyg. 51, 332–338.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings Bioinformat. 5, 150–163.

Lindsay, D.S., Dubey, J.P., 1989. Immunohistochemical diagnosis of *Neospora caninum* in tissue sections. Am. J. Vet. Res. 50, 1981–1983.

Mense, M.G., Dubey, J.P., Homer, B.L., 1992. Acute hepatic necrosis associated with a *Sarcocystis*-like protozoa in a sea lion (*Zalophus californianus*). J. Vet. Diagn. Invest. 4, 486–490.

Rakich, P.M., Dubey, J.P., Contarino, J.K., 1992. Acute hepatic sarcocystosis in a chinchilla. J. Vet. Diagn. Invest. 4, 484–486.

Resendes, A.R., Juan-Sallés, C., Almeria, S., Majó, N., Domingo, M., Dubey, J.P., 2002. Hepatic sarcocystosis in a striped dolphin (*Stenella coeruleoalba*) from the Spanish Mediterranean coast. J. Parasitol. 88, 206–209.

Romand, S., Thulliez, P., Dubey, J.P., 1998. Direct agglutination test for serologic diagnosis of *Neospora caninum* infection. Parasitol. Res. 84, 50–53.

Sahasrabudhe, V.K., Shah, H.L., 1966. The occurrence of *Sarcocystis* sp. in the dog. J. Protozool. 13, 531.

Trasti, S.L., Dubey, J.P., Webb, D.M., Blanchard, T.W., Britt, J., Fritz, D., Lewis, R.M., 1999. Fatal visceral and neural sarcocystosis in dogs. J. Comp. Pathol. 121, 179–184.

Vashisht, K., Lichtensteiger, C.A., Miller, L.A., Gondim, L.F.P., McAllister, M.M., 2005. Naturally occurring *Sarcocystis neurona*-like infection in a dog with myositis. Vet. Parasitol. 133, 19–25.

Yantis, D., Moeller, R., Braun, R., Gardiner, C.H., Aguirre, A., Dubey, J.P., 2003. Hepatitis associated with a *Sarcocystis canis*-like protozoan in a Hawaiian monk seal (*Monachus schauinslandi*). J. Parasitol. 89, 1258–1260.

Zeman, D.H., Dubey, J.P., Robison, D., 1993. Fatal hepatic sarcocystosis in an American black bear. J. Vet. Diagn. Invest. 5, 480–483.